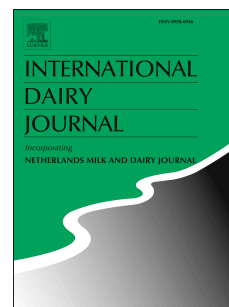


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Physicochemical properties and issues associated with trypsin hydrolyses of bovine casein-dominant protein ingredients

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ABSTRACT

Milk protein concentrate (MPC) and sodium caseinate (NaCas) were hydrolysed using the enzyme trypsin and the subsequent physical properties of the two ingredients were examined. Trypsin hydrolysis was carried out at pH 7 and at 45 °C on 11.1% (w/w) protein solutions. Heat inactivation of trypsin was carried out when the degree of hydrolysis reached either 10 or 15%. Size-exclusion chromatography and electrophoresis confirmed a significant reduction in protein molecular weight in both ingredients. However, whey proteins in MPC were more resistant to trypsin hydrolysis than casein. Oil-in-water emulsions were prepared using intact or hydrolysed protein, maltodextrin, and sunflower oil. Protein hydrolysis had a negative effect on the subsequent physical properties of emulsions, compared with non-hydrolysed proteins, with a larger particle size (only for NaCas stabilised emulsions), faster creaming rate, lower heat stability, and increased sedimentation observed in hydrolysed protein emulsions.

1. Introduction

Casein-dominant protein ingredients are widely used in dairy-based formulations due to their amphiphilic nature and good heat stability. Caseins represent ~80% (w/w) of the total nitrogen in bovine milk and are utilised in food products such as cream liqueurs, coffee whiteners, whipping creams, cheese analogues, cereal goods, artificial meats, and dietetic foods (Fox & Kelly, 2004). Milk protein concentrates (MPC) are obtained from pasteurised skimmed milk using membrane separation, namely, ultrafiltration and diafiltration followed by evaporation and spray drying (Mistry & Hassan, 1991). Diafiltration is used to obtain MPC with a protein content of $\geq 70\%$ (w/w) by further filtering out lactose and minerals present in the retentate (Mistry, 2002). MPC powders are categorised by their protein content (w/w), with the ratio of casein to whey protein remaining the same as those naturally found in skim milk (Kelly, 2011). Alternatively, sodium caseinate (NaCas), a form of water-soluble casein, is obtained by adding dilute acid or lactic acid starter cultures to skim milk until pH 4.6 is achieved, resulting in the solubilisation of calcium phosphate and precipitation of casein. With the whey separated, sodium hydroxide is then added to neutralise the acid casein resulting in the formation of NaCas with a protein content of ~90% (w/w) and approximately 1.3% (w/w) sodium content on a dry basis (Augustin, Oliver, & Hemar, 2011).

Although MPC and NaCas are highly nutritious food ingredients, cow milk allergy (CMA) generally occurs in children and is often out-grown by the age of four, although, CMA may also last for life or alternatively commence in adulthood (El-Agamy, 2007). Allergy symptoms due to CMA can be immediate as a result of Immunoglobulin E-induced reactions leading to respiratory, cutaneous or intestinal symptoms, as well as anaphylactic shock or can result in a delayed reaction that could lead to skin or intestinal discomfort (Sicherer, 2000; Taylor, 1986). Therefore, the use of milk protein hydrolysates in food

products can be beneficial in improving hypoallergenicity and ease of digestion (O'Mahony, Ramanujam, Burgher, & O'Callaghan, 2011; Sinha, Radha, Prakash, & Kaul, 2007; Terracciano, Isoardi, Arrigoni, Zoja, & Martelli, 2002;) as well as providing compounds with useful biological activity (Fekete, Givens, & Lovegrove, 2015; Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007; Sakana, Tachibana, Ishihara, & Juneja, 2005). Pre-digested milk proteins have beneficial properties such as lowering of gastrointestinal irritation and have better gut digestibility and adsorption compared with intact proteins (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014).

Hydrolysis of milk protein is often achieved using proteolytic enzymes namely trypsin, papain, pepsin, chymotrypsin or subtilisin (Banach, Lin, & Lamsal, 2013; Carreira et al., 2004; Hong et al., 2015; Rajarathnam, Nongonierma, O'Sullivan, Flynn, & FitzGerald, 2016), with the amount of peptide bonds cleaved during the hydrolysis process determining the degree of hydrolysis (DH) (Adler-Nissen, 1986). Controlled hydrolysis of casein and whey proteins using enzymes results in a mixture of hydrolysed protein, unhydrolysed protein, and low molecular weight material (Kilara & Chandan, 2011).

The use and effective delivery of pre-digested milk protein ingredients in complete nutritional formulations means their incorporation in to complex emulsion systems. Oil-in-water (O/W) emulsions are an integral part of the macronutrient composition of specialised nutrition formulations, with the utilisation of hypoallergenic and high-tolerance formulae for clinical and infant nutrition purposes produced with milk protein hydrolysates (Schröder, Berton-Carabin, Venema, & Cornacchia, 2017; Schröder et al., 2017). Milk protein hydrolysates have been used successfully as an emulsifier to form O/W emulsion based model systems in numerous studies (Drapala, Auty, Mulvihill, & O'Mahony, 2016; Euston, Finnigan, & Hirst, 2001; Schröder et al., 2017; Sinha, Radha, Prakash, & Kaul, 2007). However, Singh and Dalglish (1998) reported that whey protein hydrolysates with a DH of

more than 28% have sub-standard emulsifying ability, with emulsification capacity greatest at 10–20% DH. According to Chobert, Bertrand-Harb, and Nicolas (1988), hydrolysates should have a molecular mass distribution of above 5 kDa to maintain its emulsifying property. Good emulsifying properties have been found in peptides with a chain length of 20 amino acids or more (Lee, Shimizu, Kaminogawa, & Yamauchi, 1987; Chaplin & Andrew, 1989). It has been suggested that the peptide length has less of an impact on emulsifying ability compared with the hydrophobic/hydrophilic sequence of the amino acids that form the peptides (Rahali, Chobert, Haertle, & Gueguen, 2000).

Numerous studies have reported work on whey protein hydrolysis and their use in subsequent emulsion systems (Drapala et al., 2016; Schröder et al., 2017). However, studies on hydrolysis of micellar casein and caseinates with the goal of forming stable O/W emulsions have been limited. The use and interest in using casein hydrolysates is becoming more industrially relevant, mainly due to the rapid development and expansion of whey protein production, and thus the significant development of both micellar and non-micellar casein streams. The significant production of casein needs to be absorbed and new uses for these ingredients are being sought. Uses in specialised infant formulas and other medical nutritional products add high-value to these ingredients, but there are significant processing challenges for industry. The current study aimed to highlight some of these issues and add to the limited previous knowledge on casein hydrolyses. Therefore, the aim of this study was to develop emulsions using casein-based hydrolysates from MPC and NaCas and to examine their heat stability, emulsification and rheological properties.

2. Materials and methods

2.1. Materials

Milk protein concentrate (MPC) and sodium caseinate (NaCas) were obtained from local commercial dairy ingredient suppliers. Maltodextrin (Glucidex 29) was obtained from Roquette UK Ltd (Corby, UK). Trypsin (Trypsin 250) was attained from Biocatalysts Limited (Wales, UK) and sunflower oil was sourced from a local supermarket.

2.2. Preparation of milk protein concentrate and sodium caseinate hydrolysates

MPC and NaCas powders were dispersed and stirred in deionised water (11.1%, protein basis, w/w, in water) at 50 °C for 2 h and left overnight at 4 °C. Subsequently, ultrasonication of the dispersions was carried out using an ultrasound device (Hielscher UIP1000hd, Hielscher Ultrasonics GmbH, Warthestraße 21 D-14513, Berlin, Germany), fitted with a 22 mm diameter BS2d22 (F) sonotrode for 30 s at 100 kHz to ensure complete hydration. Hydrolysis was carried out using trypsin at pH 7.0 (45 °C). An enzyme: protein ratio of 1:50 and 1:10 was applied to achieve a 10 and 15% DH, respectively. Protein dispersions were stirred with an over-head stirrer at 300 rpm and the pH was maintained at pH 7.0 with 1 N NaOH using a Metrohm 842 Titrando pH stat dosing unit (Metrohm Ltd., Herisau, Switzerland) with Metrohm TiAMO software. The DH is described as the number of peptide bonds cleaved (h) over the number of total peptide bonds (h_{tot}), as described in equation (1) (Adler-Nissen, 1986):

$$\% \text{ DH} = 100 \times B \times N_B \times \frac{1}{\alpha} \times \frac{1}{\text{MP}} \times \frac{1}{h_{tot}} \quad (1)$$

where B is the volume of base solution, N_b is the normality of the base, $1/\alpha$ is the average dissociation degree of α -NH groups (2.71 at pH 7.0 and 45 °C), MP is the molecular mass of protein and h_{tot} is the number of peptide bonds in the protein substrate (i.e., MPC = 8.32 and casein = 8.2).

Enzymatic hydrolysis was terminated by heating the protein dispersions in a water bath at 85 °C for 20 min. The pH stat dosing unit was also used to adjust unhydrolysed protein dispersions (MPC and NaCas) to pH 7.0 using 1 N NaOH at 45 °C followed by heating in a water bath at 85 °C for 20 min.

2.3. Protein and protein hydrolysate analysis

2.3.1. Electrophoresis

Electrophoresis was carried out using NuPAGE® bis-Tris electrophoresis system (Life Technologies, Carlsbad, California, USA), under reducing conditions. Protein dispersions were diluted to 3 µg µL⁻¹ with Milli-Q water and mixed with 2.5 µL NuPAGE® LDS sample buffer (4×) and 1 µL NuPAGE® reducing agent (10×). The mixtures were then heated for 10 min at 80 °C using an incubator. Protein samples and a molecular mass standard (Mark12™ Unstained Standard, Thermo Fisher Scientific Inc.) were loaded into pre-casted NuPAGE® 4-12% gradient bis-Tris gel with NuPAGE® MOPS SDS running buffer. Electrophoresis was run at in XCell SureLock™ Mini-Cell with constant voltage of 200 V for 50 min. Gels were stained overnight in a solution of 0.5% (w/w) Coomassie Blue R250, 25% (w/w) isopropanol and 10% (w/w) acetic acid. This was followed by de-staining with a solution of 10% isopropanol and 10% acetic acid until a clear background was achieved.

2.3.2. Size exclusion chromatography

Size exclusion chromatography (SEC) was carried out as described by O'Loughlin et al. (2013) with slight modifications. The protein dispersions were diluted with Milli-Q water to a concentration of 2.5 mg mL⁻¹ and filtered through 0.22 µm low protein binding membrane filters (Sartorius Stedim, Surrey, UK). The sample (20 µL) was injected on to a

HPLC system, Waters 2695 separation module and a Waters 2487 dual wavelength absorbance detector (Milford, MA, USA) at 214 nm equipped with a TSK Gel G2000SW and a 7.8 mm × 600 mm column (Tosoh Bioscience GmbH, Stuttgart, Germany). An isocratic gradient mobile phase of 30% acetonitrile and 0.1% TFA (% , v/v) was used and the samples separation was run for 60 min at 0.5 mL min⁻¹. The mobile phase was filtered through a 0.45 µm high velocity filters (Millipore (UK) Ltd., Durham, UK) under vacuum prior to usage. A molecular weight standard curve was obtained using bovine serum albumin, carbonic anhydrase, β-lactoglobulin, α-lactalbumin, aprotinin, insulin, bacitracin, dipeptide His-Leu.

2.4. Emulsion preparation

Oil-in-water emulsions were prepared using 5% (w/w) intact or hydrolysed protein, 10% (w/w) sunflower oil, and 10% (w/w) maltodextrin (Dextrose equivalent, DE, 29). Intact and hydrolysed protein dispersions (11.1%, w/w, protein basis in water) were added into sunflower oil. Corn syrup (Glucidex 29, Roquette UK Ltd, Corby, UK) was dissolved in deionised water (25%, w/w, in water) at 50 °C and added into the protein:oil mixture followed by agitation with a high-shear mixer (Ultra-Turrax, IKA, Staufen, Germany) for 30 s at 10,000 rpm. The pre-emulsion was then homogenised at room temperature using a one-stage valve homogeniser (APV Lab Series 1000 Homogenizer, SPX, Charlotte, NC, USA) for 3 passes (2 passes at 150 bar and 1 pass at 50 bar). Six different emulsions were prepared using non-hydrolysed MPC (MPC-E), non-hydrolysed NaCas (NaCas-E), hydrolysed MPC to 10% DH (HYMPC10-E) and 15% DH (HYMPC15-E), and hydrolysed NaCas to 10% DH (HYNaCas10-E) and 15% DH (HYNaCas15-E).

2.5. Particle size analysis

Particle size of emulsions was determined using a Mastersizer 3000 laser diffraction instrument (Malvern Instruments Ltd., Malvern, UK). Measurements were performed at a laser obscuration (%) of 4–6%, particle refractive index of 1.45, dispersant refractive index of 1.33 and absorption index of 0.001. Particle size distributions were reported as volume-weighted mean diameter ($D_{4,3}$) and cumulative volume diameter of 90% (D_{90}).

2.6. *Zeta-potential*

The zeta (ζ)-potential of protein-stabilised emulsions was measured using a Zetasizer (Nano-ZS90, Malvern Instruments, Worcestershire, UK). The samples were diluted in Milli-Q water at a ratio of 1:50 and placed into plastic folded capillary cells (DTS 1070, Malvern Instruments Ltd.). Measurements were carried out at 20 °C with a refractive index of 1.46 and adsorption index of 0.001.

2.7. *Emulsion stability*

Emulsion stability was measured using a LUMiSizer (L.U.M. GmbH, Berlin, Germany). Aliquots of all emulsions (0.4 mL) were transferred into polycarbonate cells (LUM 2mm, PC, Rect. Synthetic Cell 110-131××, L.U.M. GmbH) and measurements were carried out at $1878 \times g$ at 25 °C for 50 min and the resultant data obtained were analysed using SEPVIEW v.6 software (L.U.M. GmbH). LUMiSizer assess emulsion stability by determining the intensity of transmitted light across the sample length as a function of position and time. A second aliquot of all emulsions (1.5 mL) was taken and centrifuged in

Eppendorf Tubes[®] (Eppendorf 5417R, Eppendorf, Hamburg, Germany) for 20 min at 20 °C and $800 \times g$ for visual inspection.

2.8. Rheological measurements

Rheological measurement of emulsions was carried using a controlled-stress rheometer (AR-G2, TA Instruments, Crawley, West Sussex, UK) equipped with a starch pasting cell (SPC) geometry. Samples (28 g) were firstly allowed to equilibrate under no shear for 2 min at 15 °C. Subsequently, samples were held for 5 min at 15 °C at a shear rate of 15 s^{-1} , heated to 90 °C at a heating rate of 10 °C min^{-1} , held at a peak temperature for 15 min, then cooled back down to 15 °C (at a cooling rate of 10 °C min^{-1}), and finally held for 5 min at 15 °C.

2.9. Heat coagulation time of emulsions

The heat coagulation time (HCT) of emulsions was determined using an oil bath (Hettich ESP oil baths; Hettich Benelux BV). Samples (3.4 g) were transferred into glass tubes (4 mL, 120 mm length, 20 mm outer diameter, 14 mm inner diameter, Hettich Benelux BV, Geldermalsen, The Netherlands). The glass tubes were sealed with rubber bungs, fixed on a metal rack, and placed into the oil bath set at 90, 100, 120 or 140 °C at an oscillation rate of 7 oscillations min^{-1} . Heat stability was defined as the time where coagulation/flecking was observed on the wall of the glass tubes. All protein hydrolysis and emulsions were prepared in triplicate (i.e., three completely independent trials) with all data and results expressed as mean values \pm standard deviations.

3. Results

3.1. Protein and peptide profile analysis

MPC and NaCas protein dispersions were hydrolysed to 10 or 15% DH and degree of hydrolysis was determined using Eq. 1. The amount of 1 N NaOH needed to achieve HYMPC10, HYNacCas10, HYMPC15 and HYNacCas15 was 7.68, 7.57, 11.5 and 11.4 mL, respectively at 11.1% (w/w) protein content. Fig. 1 shows distinct bands of intact caseins in both MPC (lane 2) and NaCas (lane 5) at a molecular mass (M_w) of between 30 and 20 kDa under reducing SDS-PAGE conditions. Whey protein bands in the MPC sample at 18 and 14 kDa representing β -lactoglobulin and α -lactalbumin, respectively, are shown in Fig. 1, lane 2. There was a faint band at 18 kDa representing β -lactoglobulin in the NaCas sample but no α -lactalbumin (Fig. 1; lane 5). Minor whey proteins were present in both MPC and NaCas resulting in β -lactoglobulin and α -lactalbumin being observed in the gel. Hydrolysed MPC samples had no casein bands with only a faint band at 10 kDa, representing a large M_w peptide in HYMPC10 (lane 3) and HYMPC15 (lane 4). HYMPC10 (lane 3) contained a very faint band at 18 kDa (lane 3) that was completely absent at the higher DH in HYMPC15 (lane 4). Hydrolysed NaCas was found to have no protein or peptide bands on the gel at either DH (Fig. 1; lanes 6 and 7).

As SDS-PAGE provided little information on the products of hydrolysis, size exclusion chromatography (SEC) HPLC was used to measure the M_w of the peptides obtained (Fig. 2). The molecular mass of the peptides was lower than that of the intact proteins in MPC and NaCas. Two similar peaks were identified in MPC and NaCas with mass of 62.2 and 24.4 kDa as both proteins were casein-dominant. Proteins and peptides with mass of 62.2, 2.7, 1.4, 1.1, 0.56, and 0.22 kDa were obtained in both HYMPC10 and

HYNaCas10, while peptides of 1.4, 1.1, 0.56, and 0.22 kDa were found in HYMPC15 and HYNaCas15.

3.2. Particle Size

Table 1 shows the particle size values of O/W emulsions formed from intact and hydrolysed MPC and NaCas. Size distribution profiles (Fig. 3A) and D_{90} (Table 1) values of intact (D_{90} , 1.57 μm ; $D_{4,3}$, 0.58 μm) (and hydrolysed MPC were similar regardless of DH (HYMPC10-E: D_{90} , 1.75 μm ; $D_{4,3}$, 0.79 μm ; HYMPC15-E: D_{90} , 1.79 μm ; $D_{4,3}$, 0.68 μm). Emulsions stabilised using intact NaCas had a significantly larger particle size than MPC stabilised emulsions (D_{90} , 2.08 μm ; $D_{4,3}$, 0.82 μm). However, emulsions stabilised with hydrolysed NaCas had significantly ($P < 0.05$) larger particle size values with increasing DH (HYNaCas10-E: D_{90} , 2.89 μm ; HYNaCas15-E: D_{90} , 5.84 μm), as also seen by a shift to the right in particle size distribution profiles (Fig. 3B).

3.3. Emulsion Stability

Fig. 4 shows the separation profiles for all emulsions, whereby intact MPC was found to be more stable against gravitational separation compared with NaCas based emulsions. Intact and hydrolysed MPC based emulsions were generally more stable than intact and hydrolysed NaCas based emulsions when compared across the same DH. Fig. 4 also showed that emulsions stabilised with protein hydrolysates (HYMPC10-E; HYMPC15-E; HYNaCas10-E; HYNaCas15-E) had lower emulsion stability than emulsions with intact proteins (MPC-E and NaCas-E) and the former resulted in higher phase separation rates. Visual creaming and sedimentation in the emulsions after centrifugation is shown in Fig 5.

Hydrolysing MPC significantly reduced its resistance to gravitational separation, compared with intact MPC, as shown by the clear phase separation in Fig. 5. Obvious sediments can be seen in HYMPC10-E and HYNaCas10-E while only low levels of sediment were seen in HYMPC15-E and HYNaCas15-E.

3.4. ζ -potential

Table 1 shows the ζ -potential values of emulsions made both from intact and hydrolysed proteins. All emulsions were highly negatively charged at pH 7 as they were well above the isoelectric point (pI) of casein ($pI \sim \text{pH } 4.6$) and whey protein ($pI \sim \text{pH } 5.0$). Interestingly, the ζ -potential of intact MPC stabilised emulsion (-28.2 mV) was significantly ($P < 0.05$) lower than intact NaCas emulsion (-68.2 mV). This remained the case when comparing hydrolysates obtained from MPC and NaCas (HYMPC10-E: -29.07 mV ; HYNaCas10-E: -43.67 mV ; HYMPC15-E: -32.2 mV ; HYNaCas15-E: -56.2 mV). However, the ζ -potential of MPC was not significantly ($P > 0.05$) affected by the DH, unlike NaCas where the ζ -potential significantly decreased after hydrolysis (Table 1).

3.5. Rheological properties of emulsions

The viscosity of emulsions as a function of temperature is shown in Fig. 6. The apparent viscosity of all systems was found to decrease with increasing temperature which is a well-established occurrence. However, it was noted that there was a sudden increase followed by decrease in the viscosity of the hydrolysate based emulsions (HYNaCas10-E; HYNaCas15-E; HYMPC15-E) upon heating to between 60 and 70 °C, which may be the

onset of emulsion destabilisation. Drapala et al. (2016) found similar results during the heating of hydrolysed whey protein emulsions.

Initial viscosity values of emulsions prepared with either MPC (27.1 mPa s) or NaCas (35.1 mPa s) were higher than those prepared using hydrolysed proteins (HYMPC10-E: 21.53 mPa s; HYMPC15-E: 23.35 mPa s; HYNaCas10-E: 20.18 mPa s; HYNaCas15-E: 21.05 mPa s). Interestingly, the apparent viscosity of MPC-E was lower after heating to 90 °C and cooling to 15 °C compared with its initial viscosity; while apparent viscosity of NaCas-E remained the same after heat treatment. Apparent viscosity of NaCas-E was also found to be higher than other systems. However, there was an increase in the apparent viscosity of HYMPC10-E (29.6 mPa s), HYMPC15-E (31.6 mPa s), HYNaCas10-E (26.9 mPa s), and HYNaCas15-E (24.9 mPa s) post-heating at 90 °C with a higher increase observed in HYMPC10-E and HYMPC15-E. The hydrolysates based emulsions were still free flowing and did not gel upon heating although the presence of flecks or aggregates was observed.

3.6. Heat coagulation time

Table 2 shows the heat coagulation time (HTC) of emulsions at temperatures of 90, 100, 120 or 140 °C. All emulsions formed using hydrolysates were found to have low heat stability with heat coagulation times of not more than 100 s at 90, 100 or 120 °C. Heat coagulation time of hydrolysate-based emulsions and MPC-E were not determined at 140 °C due to the low heat stability at this temperature. As HCT using the oil bath method is relatively subjective; only differences of a few minutes or more can be quantified and reported as significant while differences by a number of seconds were deemed not possible. Intact proteins were able to form heat stable emulsions with a heat coagulation time of more than 1800 s at 90 and 100 °C for MPC, and 90, 100 and 120 °C for NaCas. However, the

intact MPC emulsion was not heat stable at 140 °C, compared with the intact NaCas emulsion which remained heat stable at 140 °C for ~900 s.

4. Discussion

Hydrolysed milk proteins can be used as an alternative dairy nutritional source for individuals who suffer from cow milk allergy (CMA). Kilara and Chandan (2001) stated that peptides produced for hypoallergenic purposes should be extensively hydrolysed and contain peptides with mass less than 5 kDa with 90% of the peptides at approximately 500 Da while less-extensively hydrolysed peptides will be sufficient for supplementation purposes. In the present study, peptides with similar mass were obtained from hydrolysis of MPC and NaCas mainly due to the casein-dominant nature of both proteins and therefore containing similar enzymatic cleavage sites for trypsin activity.

However, a slower shift in the number of peptides with high Mw to lower Mw was observed in MPC, compared with NaCas at the same DH based on the chromatogram peaks (Fig. 2) and may be due to slower enzymatic action. Guo, Fox, Flynn, and Kindstedt (1995) stated that β -lactoglobulin, a globular protein present in MPC, was less susceptible to enzymatic hydrolysis compared with casein due to significant structure differences. It was also noted that increasing the DH from 10 to 15% did not further reduce the mass of the peptides to below 0.22 kDa. Increasing the DH was found to only shift the mass of the peptides from higher to lower Mw where a loss or reduction in the peak of high mass peptides was observed with concomitant increase in the area peaks of lower mass peptides. This may be attributed to the depletion of specific enzyme cleavage sites on the proteins. Enzyme specificity is one of the main factors in enzymatic hydrolysis meaning the enzyme cleaves at a specific location or cleaves a number of peptide linkages occurring either

sequentially to give one peptide at a time or through the zipper mechanism; by producing intermediate sized peptides followed by further hydrolysis into smaller peptides (Kilara & Chandan, 2001). Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residues. If a proline residue is on the carboxyl side of the cleavage site, trypsin mediated cleavage will not occur. If an acidic residue is on either side of the cleavage site, the rate of trypsin hydrolysis has been shown to be slower. Barry, Dinan, and Kelly (2017) found similar results when hydrolysing buttermilk; where an increase in DH from 9 to 25% did not significantly change the molecular mass distribution, as only the peaks of smaller peptides obtained initially increased and no new peaks of lower mass peptides were formed with increasing DH. Banach et al. (2013) also reported that the DH of MPC80 did not further increase with increasing the hydrolysis time from 5 to 10 min using chymotrypsin and from 10 to 60 min using trypsin as a result of absence of specific cleavage sites.

The use of hydrolysed proteins in nutritionally complete products requires them to retain some of their original functional properties, particularly emulsifying capability. The fact that hydrolysed MPC was capable of stabilising smaller oil droplets compared with hydrolysed NaCas may be due to the presence of low levels of intact whey protein. However, even hydrolysed whey proteins can produce stable emulsions due to the exposure of thiol, disulphide, and non-polar groups, capable of forming multilayers at the oil-water interface. Overall, the intact proteins were able to form emulsions with smaller oil droplets compared with their corresponding hydrolysates (Fig. 3). However, increasing the DH of NaCas from 10 to 15% further reduced its emulsifying ability as seen by an increase in particle size ($D_{(4,3)}$) from 1.10 μm to 6.02 μm (Table 1). Several studies (Mahmoud, Malone, & Cordle, 1992; van der Ven, Gruppen, de Bont, & Voragen, 2001) found that the emulsifying activity of casein decreased with increasing DH; while Rahali et al. (2000) stated that the length of peptides has a lesser impact on emulsifying capacity than the hydrophilic/hydrophobic

sequence of the peptides. The increased particle size of emulsions stabilised with hydrolysed casein also contributed to their subsequent rapid creaming rate (Fig. 4). This was due to differences in the size of the oil droplets formed, as described by Stokes' Law, with smaller oil droplets obtained using intact MPC and NaCas. Interestingly, increasing the DH of MPC and NaCas had little effect on the creaming rate (Fig. 4). According to Chobert et al. (1988), hydrolysates should have a molecular mass distribution of above 5 kDa to maintain good emulsification properties. However, to achieve hypoallergenicity the molecular mass of peptides need to be below 5 kDa (Kilara & Chandan, 2001), leading to a balance between good emulsification capacity and achieving the correct nutritional benefits of the casein hydrolysates. Therefore, using lower DH values may improve emulsification properties but might not reduce the risk of allergenicity.

As well as compromised emulsion quality from hydrolysed proteins, the heat stability was also greatly reduced (Table 2). Studies have shown whey protein hydrolysates to have improved heat stability but this does not readily translate to emulsion systems (Singh & Dalgleish, 1998). The lower molecular mass peptides are unable to provide sufficient stability when exposed to high temperatures and similar to the mechanisms involved in providing emulsion instability the decrease in steric hindrance results in a significant increase in heat sensitivity. Drapala et al. (2016) found similar results with emulsions stabilised using whey protein hydrolysates, but showed that conjugation with maltodextrin significantly improved heat stability. This may be a suitable option for some nutritional products which contain hydrolysed dairy protein and low levels of lactose. Emulsions with intact protein were found to be more heat stable than emulsions formed using hydrolysates with both MPC-E and NaCas-E having heat coagulation times of more than 1800 s at 100 and 120 °C, respectively. The lower heat stability of the intact MPC compared with NaCas may be due to the presence

of the major whey proteins in the MPC and to a lesser extent could be attributed to the lower ζ -potential (Table 1).

5. Conclusions

Hydrolyses of casein dominant protein ingredients using trypsin was effective up to 15% DH, further hydrolyses was hindered due to a lack of available substrate cleavage sites. The production of hydrolysed casein emulsions proved to be difficult, with heat stability significantly reduced compared with the original intact protein ingredients. However, MPC hydrolysates proved far more effective at stabilising small oil droplets compared with NaCas for both 10 and 15% DH, due to the presence of intact and hydrolysed whey proteins. Some improvements may be found by increasing the DH from 10 to 15% which reduced sedimentation in emulsions but did not improve heat stability. To improve the stability of casein dominant hydrolysates, a number of possible further studies could be examined mainly using protein-carbohydrate conjugation, interfacial structuring and pre-heat treatment of peptides to improve heat stability of hydrolysate based beverages. The use of enzyme mixtures (trypsin, pepsin, papain, alcalase, etc.) to achieve higher levels of hydrolyses may also be investigated. If excessive hydrolyses is not required for hypoallergenicity purposes, but for improved digestibility, then lower levels of hydrolyses (DH < 10%) could be examined that might improve emulsification capacity. Improving emulsion stability could also be investigated using non-protein emulsifiers in conjunction with casein hydrolysates. Also of interest might be the inclusion of casein hydrolysates in to other products, such as protein bars, yoghurts, nutritional shakes, etc.

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Figure legends

Fig. 1. SDS-PAGE protein profile patterns of milk protein concentrate (MPC), sodium caseinate (NaCas), and its hydrolysates at 10% (HYMPC10; HYNaCas10) and 15% (HYMPC15; HYNaCas15) degree of hydrolysis (DH) under reducing condition (lane 1, molecular mass marker; lane 2, MPC; lane 3, HYMPC10; lane 4, HYMPC15; lane 5, NaCas; lane 6, HYNaCas10; lane 7= HYNaCas15).

Fig. 2. Retention time (min) and molecular mass (Da) data of (a) milk protein concentrate (MPC; top trace) and hydrolysates of MPC at 10 and 15% degrees of hydrolysis (DH) (HYMPC10 and HYMPC15; middle and bottom traces, respectively) and (b) sodium caseinate (NaCas; top trace) and NaCas hydrolysates at 10 and 15% DH (HYNaCas10 and HYNaCas15; middle and bottom traces, respectively).

Fig. 3. Particle size distribution profiles of emulsions formed using (a) milk protein concentrate (MPC-E; solid line) and hydrolysates of MPC at 10 and 15% degree of hydrolysis (DH) (HYMPC10-E and HYMPC15-E; dashed line and dotted line, respectively) and (b) sodium caseinate (NaCas-E; solid line) and NaCas hydrolysates at 10 and 15% DH (HYNaCas10-E and HYNaCas15-E; dashed line and dotted line, respectively).

Fig. 4. Phase separation profiles of emulsion stabilised using milk protein concentrate (MPC-E) (a), sodium caseinate (NaCas-E) (c), and MPC and NaCas hydrolysates at 10% (HYMPC10-E (b); HYNaCas10-E (d)) and 15% (HYMPC15-E (c); HYNaCas15-E (e)) degree of hydrolysis (DH).

Fig. 5. Photographic image of emulsions stabilised with milk protein concentrate (MPC-E), sodium caseinate (NaCas-E), and hydrolysates of MPC and NaCas at 10% (HYMPC10-E; HYNaCas10-E) and 15% (HYMPC15-E; HYNaCas15-E) degree of hydrolysis (DH) after centrifugation at 800g for 20 minutes.

Fig. 6. Apparent viscosity (dotted lines) of oil-in-water emulsions stabilised with milk protein concentrate (MPC-E) (a), sodium caseinate (NaCas-E) (d), and MPC and NaCas hydrolysates at 10% [HYMPC10-E (b); HYNaCas10-E (e)] and 15% [HYMPC15-E (c); HYNaCas15-E (f)] degree of hydrolysis, as a function of temperature (solid lines).

Table 1

Particle size (D_{90} and $D_{4,3}$) and ζ -potential (mV) data of emulsions stabilised by milk protein concentrate (MPC-E), sodium caseinate (NaCas-E), and hydrolysates of MPC and NaCas at 10% (HYMPC10-E; HYNaCas10-E) and 15% (HYMPC15-E; HYNaCas15-E) degree of hydrolysis (DH).

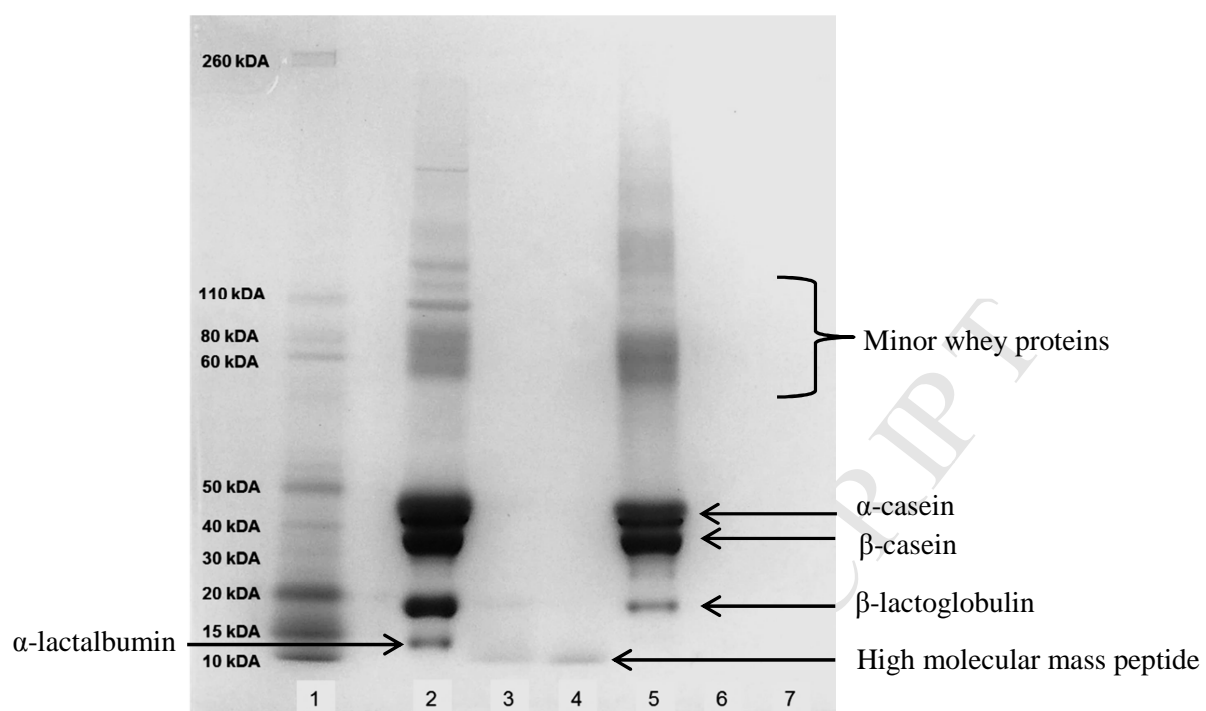
Sample	D_{90} (μm)	$D_{(4,3)}$ (μm)	ζ -potential (mV)
MPC-E	1.57 ± 0.38	0.58 ± 0.15	-28.20 ± 0.85
HYMPC10-E	1.75 ± 0.02	0.79 ± 0.14	-29.07 ± 5.79
HYMPC15-E	1.79 ± 0.07	0.68 ± 0.01	-32.20 ± 0.98
NaCas-E	2.08 ± 0.02	0.82 ± 0.04	-68.20 ± 2.04
HYNaCas10-E	2.89 ± 0.39	1.10 ± 0.04	-43.67 ± 5.07
HYNaCas15-E	5.84 ± 0.95	6.08 ± 5.53	-56.27 ± 2.74

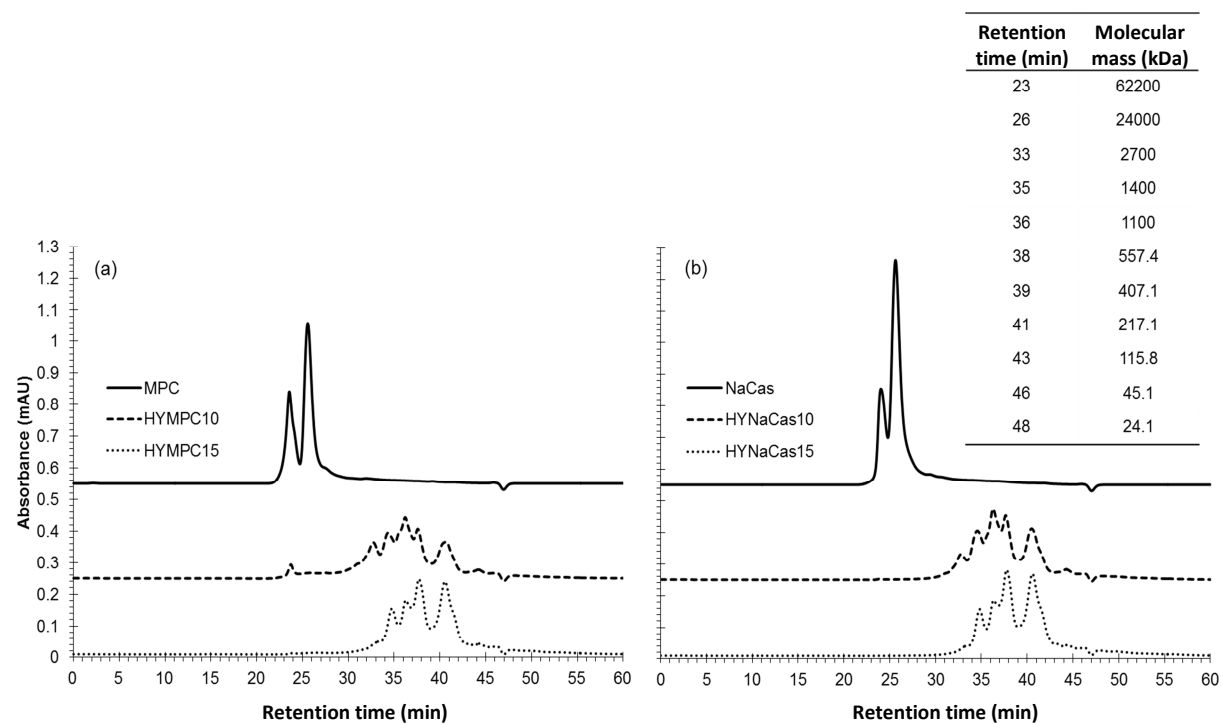
Table 2

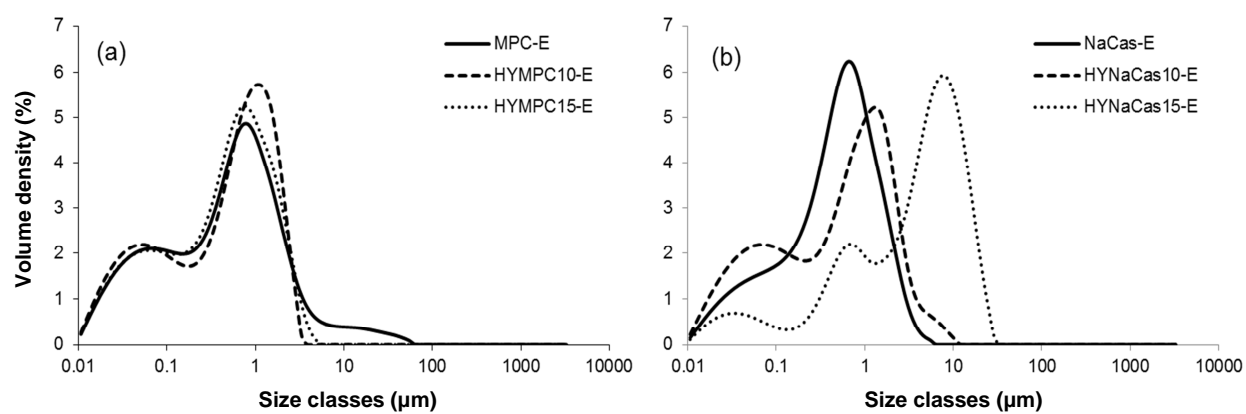
Heat coagulation time (s) of emulsions stabilised with milk protein concentrate (MPC-E), sodium caseinate (NaCas-E), and hydrolysates of MPC and NaCas at 10% (HYMPC10-E; HYNaCas10-E) and 15% (HYMPC15-E; HYNaCas15-E) degree of hydrolysis (DH) as a function of temperature at 90, 100, 120 and 140 °C. ^a

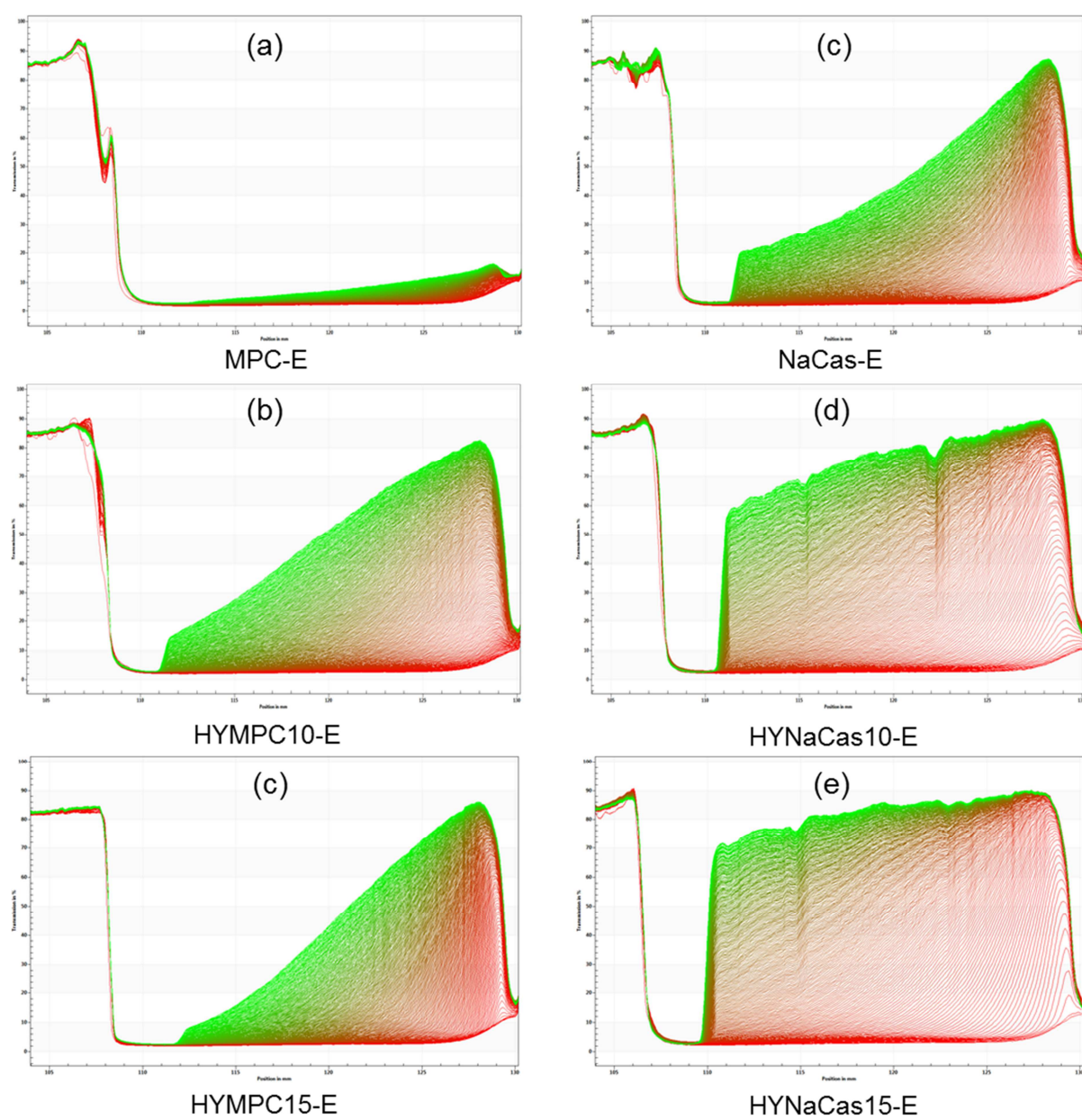
Samples	Degree of hydrolysis (%)	Heat coagulation time (s)			
		90 °C	100 °C	120 °C	140 °C
MPC	0	>1800 ^c	>1800 ^c	60 ^b	-
	10	65 ^a	40 ^a	-	-
	15	68 ^a	60 ^a	35 ^a	-
NaCas	0	>1800 ^c	>1800 ^c	>1800 ^c	900 ^b
	10	58 ^a	45 ^a	-	-
	15	95 ^a	60 ^a	35 ^a	-

^a Values in a column followed by a different superscript letter are significantly different ($P < 0.05$); a dash indicates that the samples coagulated immediately.

**Figure 1.**

**Figure 2.**

**Figure 3.**

**Figure 4.**

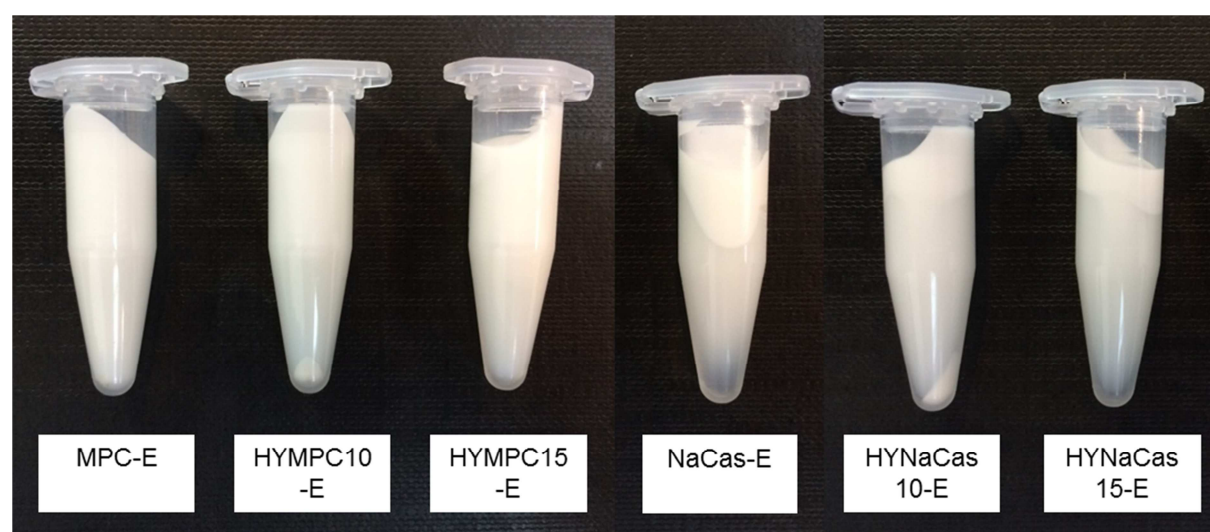
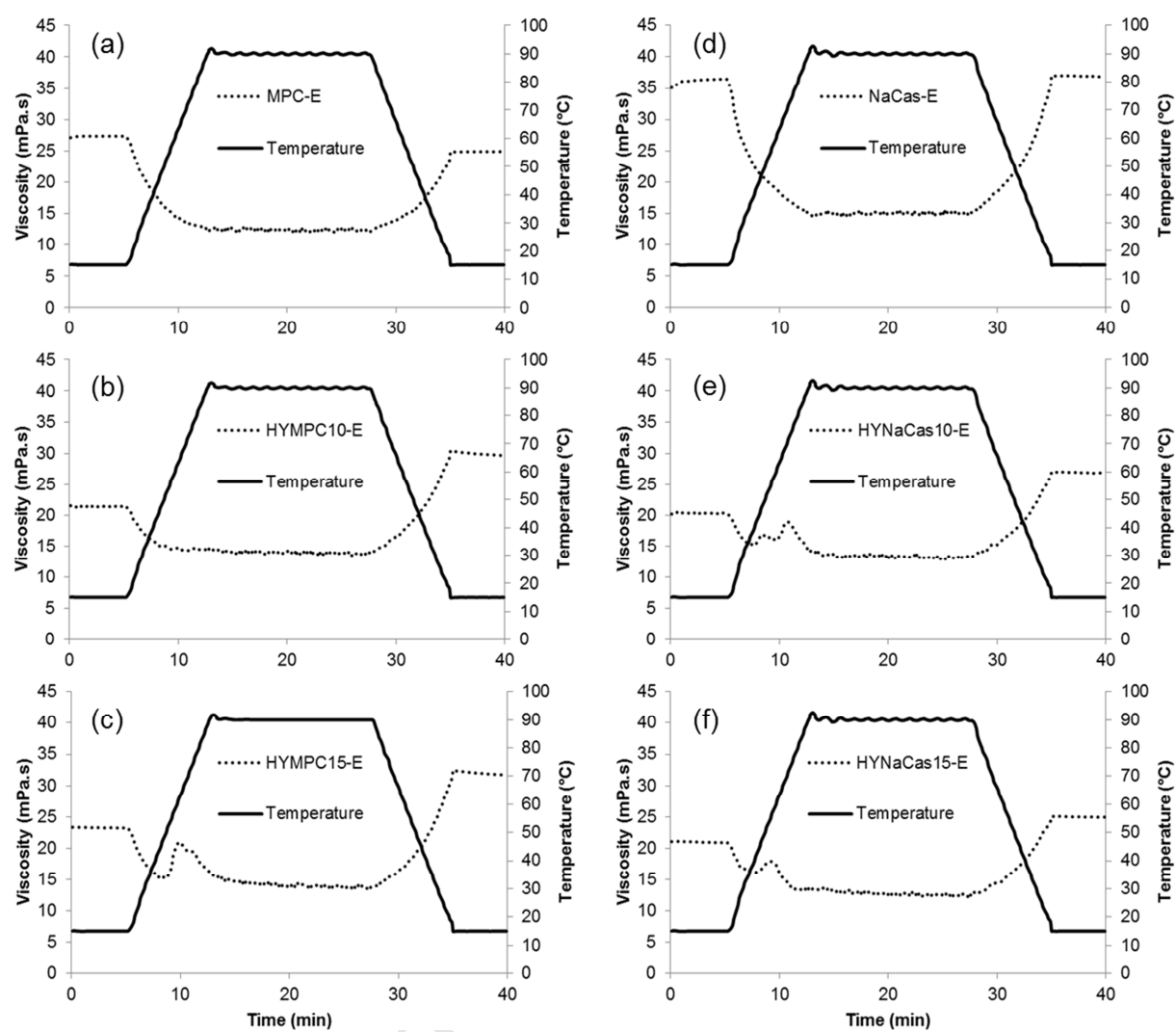


Figure 5.

**Figure 6.**